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Neurogenetics of Pelizaeus-Merzbacher disease

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Abstract

Pelizaeus-Merzbacher disease (PMD) is an X-linked disorder caused by mutation in the PLP1 gene, which encodes the proteolipid protein of myelinating oligodendroglia. PMD exhibits phenotypic variability that reflects its considerable genotypic heterogeneity, but all forms of the disease result in central hypomyelination associated with early neurological dysfunction, progressive deterioration, and ultimately death. PMD may present as a connatal form, or as less severe classical and transitional forms, the latter including spastic paraplegia type 2. These disorders may be associated with duplications, as well as with point, missense and null mutations within the PLP1 gene. A number of clinically similar Pelizaeus-Merzbacher-like disorders (PMLD) may also be considered in the differential diagnosis of PMD, the most prominent of which is PMLD-1, caused by mis-expression of the GJC2 gene encoding connexin-47. No effective therapy for PMD exists. Yet as a relatively pure CNS hypomyelinating disorder, with limited involvement of the PNS and relatively little attendant neuronal pathology, PMD is an attractive therapeutic target for neural stem cell and glial progenitor cell transplantation, efforts at which are now underway in a number of research centers.

Keywords

myelin; hypomyelination; leukodystrophy; remyelination; glia; oligodendrocyte

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A. Pelizaeus-Merzbacher Disease: A brief history

Pelizaeus-Merzbacher disease (PMD, MIM 312080) was first described by Friedrich Pelizaeus (Pelizaeus, 1885) and later by Ludwig Merzbacher (Merzbacher, 1910), in children presenting with nystagmus, spastic quadriparesis, ataxia and cognitive impairment. Those initial reports described 14 affected members of a single family, 12 of whom were males, and served to define the X-linked basis of PMD transmission: Children inherit PMD exclusively from their mothers. Early linkage studies assigned PMD to the X chromosome at the Xq21.3-q22 position (Mattei et al., 1986), and later studies linked it to the proteolipid

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protein (PLP) locus (Barkovich and Patay, 2012; Boespflug-Tanguy et al., 1994). We now know that PMD comprises a relatively broad spectrum of disorders, whose cardinal shared feature is dysregulation of PLP expression and/or structure. Together, their diagnosed prevalence ranges from 1:200,000–1:500,000 in the US, with international incidence ranging from 1:90,000–1:750,000 live births, depending upon the demographic (Hobson and Kamholz, 2013).

B. Major clinical phenotypes of PMD

PMD is clinically heterogeneous (Figure 2). In its prototypic form, it is characterized by pendular nystagmus, head tremor, and systemic hypotonia; with time, affected patients manifest some combination of mental retardation, choreoathetosis, dystonia, cerebellar ataxia and long tract signs, especially corticospinal dysfunction (Hudson et al., 2004; Powers, 2004). Clinically, PMD has been classified into three subtypes, according to the age of presentation (Seitelberger et al., 1996): type I, or classic PMD; type II, or connatal PMD; and type III, or transitional PMD.

Classic PMD presents before the first year of age, with nystagmus, slowly acquired or unachieved motor milestones, and significant axial hypotonia. Appendicular spasticity and involuntary movements both follow this initial presentation. The nystagmus is characterized by rapid and irregular oscillations of variable amplitude, in both horizontal and vertical directions (Lyon et al., 1996), and can be accompanied by head tremor. Optic atrophy and seizures may occur later in the course, although seizures are uncommon, and typically treatable. Yet despite this presentation, the cognition and speech of children with classic PMD can be preserved to a remarkable degree. In addition, despite their severe motor handicap, many affected children can develop the ability to ambulate with assistance. The progression of disease slows by the end of the first decade, and the life spans of affected patients vary from adolescence to young adulthood.

Connatal PMD presents earliest, in the neonatal period, and is the most aggressive of PMD phenotypes. Babies with connatal PMD manifest extrapyramidal signs, laryngeal stridor, feeding difficulties and optic atrophy. These infants are also characterized by extreme neonatal hypotonia, that may be so severe as to mimic spinal muscular atrophy (Kaye et al., 1994). They rarely if ever achieve ambulation or develop speech, and their survival is limited. In addition, these patients may develop seizures, though these are typically responsive to antiepileptic agents.

Transitional PMD combines clinical features of both the classic and connatal forms, and includes two principal phenotypes, spastic paraplegia and PLP1 null disease (Garbern et al., 2002a; Shy et al., 2003). Spastic paraplegia type 2 (SPG2) was initially described in 1994, and expanded the phenotype of PLP1 related disorders (Saugier-Veber and Munnich, 1994). It is of later onset than the classic PMD, and is characterized by spasticity of the lower extremities that can be isolated, or co-exist with varying degrees of cognitive impairment, nystagmus, ataxia, dysarthria and spastic urinary bladder. The PLP1 null phenotype represents another syndrome later described by Garbern and colleagues, and is characterized

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by complicated spastic paraplegia, with mild to moderate demyelinating peripheral neuropathy and axonal injury (Garbern et al., 1997; Garbern et al., 2002b).

C. Neuropathology and neuroradiology of PMD

Neuropathology

The brains of affected PMD patients typically show overt evidence of cerebral and cerebellar atrophy, most prominently so in the early-onset forms of the disease. There is a marked deficiency of myelin, particularly in deeper cerebral structures, but relative myelin preservation in areas surrounding blood vessels, providing the classic tigroid appearance of PMD histopathology (Boulloche and Aicardi, 1986; Zeman et al., 1964). Oligodendrocytes are dramatically reduced in number (Watanabe et al., 1973), while astrocytic numbers are increased (Seitelberger, 1970). Although not a universal finding, axonal pathology has been described in some cases (Garbern et al., 2002a), as has peripheral neuropathy (Garbern et al., 1999b). Biochemical analysis in some patients has suggested that besides PLP, that other myelin proteins may also be decreased, including myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and 2’,3’-cyclic nucleotide-3’-phosphodiesterase (CNP). Similarly, a decrease in myelin lipids (cerebroside, sulphatides and sphingomyelin) has also been reported (Bourre et al., 1978). Interestingly, while PMD is not typically thought of as being attended by significant inflammation, microglial activation has been reported in biopsies of patients with both PLP1 missense mutations and duplications, as well as in animal models with similar mutations (Southwood et al., 2013), suggesting that the innate immune system is indeed activated in the setting of disease progression.

Neuroradiology

Computed tomography (CT) first revealed that PMD patients manifest attenuation of the white matter with progressive atrophy, indistinguishably so from a number of other white matter disorders (Barkovich and Raybaud, 2012). MRI subsequently revealed overt hypomyelination, as reflected by the failure of PMD patients to develop the expected developmental increase in T1 and decrease in T2 signals characteristic of myelin maturation. These children appear to suffer a defect in myelin formation, rather than accelerated loss; there is no evidence to date of white matter destruction in PMD (Barkovich and Raybaud, 2012) (Valk and van der Knaap, 1989). As such, in connatal PMD, there is a profound lack of myelin (Inoue et al., 1997; Takanashi et al., 1999), and the typical milestones of neonatal myelination, including myelination of the posterior limb of the internal capsule and middle cerebellar peduncle, are usually not achieved (Barkovich and Raybaud, 2012). In the less severe classic form of PMD, high T1 signal is usually seen in the posterior limb of the internal capsule, as well as in the optical radiations and corona radiata, all suggesting a degree of developmentally-appropriate myelination. Nonetheless, these children manifest the near absence of low signal intensity in T2-weighted images supratentorially, and their white matter volume slowly falls over time (Valk and van der Knaap, 1989) (Figure 1). Indeed, even when initial MR imaging has appeared grossly normal, serial exams have shown the lack of developmental myelination over time, with enlargement of the cortical sulci (Barkovich and Raybaud, 2012), and concurrent cerebellar hypoplasia in some cases (van der Knaap and Valk, 2005).

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The degree of disability has been linked to the extent of radiographically-defined white matter atrophy in patients spanning a range of genotypes (Laukka et al., 2013). Diffusion tensor imaging of a group of patients with a variety of point mutations, and correspondingly diverse disease phenotypes, manifested increased apparent diffusion coefficients and perpendicular diffusion, reflecting abnormalities in myelin structure, in all patients. In contrast, irregularities in axonal structure and fasciculation, as reflected by increased parallel diffusion, were noted in only the most severe cases (Laukka et al., 2014). A correlation between MRI and histopathology, with myelin staining by Luxol fast blue, revealed that areas of low T1 signal corresponded to those areas of diminished staining that characterized hypomyelination. Similarly, areas of high signal on T2 FLAIR imaging mapped to areas of white matter fibrillary astrogliosis, as defined histologically by Holzer staining (Komaki et al., 1999).

Magnetic resonance spectroscopy (MRS) has proven more variable and less predictive than MRI-based determinations of white matter structure (Pizzini et al., 2003; Takanashi et al., 1997). Low N-acetylaspartate (NAA) peaks are most commonly reported in PMD (Bonavita et al., 2001; Garbern et al., 2002a; Pizzini et al., 2003), and may reflect neuronal loss, although normal (Takanashi et al., 1997) or even increased NAA peaks have also been described (Takanashi et al., 2002); the latter observation possibly reflecting an increase in the number of axons per voxel occasioned by the local drop in myelin sheath numbers and thickness. In other MRS studies, a low choline peak has been described in PMD, presumably reflecting the decrease in myelin proteins and lipids, and yet here also a normal peak has been noted in other cases (Takanashi et al., 2002; Takanashi et al., 1997). In regards to neurophysiological testing, brain auditory-evoked responses and somatosensory-evoked potentials (SSEPs) may each be abnormal in PMD, particularly so in those children with prominent brainstem involvement. In addition, electrophysiological evidence of peripheral neuropathy has been noted in some cases (Kaye et al., 1994; Shy et al., 2003), which have variably manifested axonal as well as demyelinating pathology.

D. Structure and function of the proteolipid protein

The proteolipid protein gene (PLP1) is a 17 kb gene with 7 exons, which maps to the proximal long arm of the X chromosome (Xq21-q22) (Mattei et al., 1986; Willard and Riordan, 1985). Its product, proteolipid protein, together with its alternate splice product DM20 protein (Nave et al., 1987), is one of the most abundant protein constituents of myelin. Indeed, the importance of PLP to myelin is suggested by its abundance: together with myelin basic protein, PLP constitutes 60–80% of all myelin protein content (Quarles and Macklin, 2006).

Both PLP1 and DM20 are both tetraspanins, formed by alternative splicing at one of two 5' splice donor sites in exon 3, which join the same splice acceptor site (Nave et al., 1987). The proteins have four transmembrane domains, two extracellular loops containing two cysteine bridges, one cytosolic loop and cytosolic N and C termini (Figure 3). The proteolipid protein - which was first discovered, and has also been known, as Folch-Lees protein (Folch and Lees, 1951) - has 276 amino acids, and differs from DM20 by a 35 amino acid segment in its cytosolic loop. PLP apposition to the oligodendrocyte membrane has suggested its role in

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forming the double-spaced intraperiod line (IPL), and thus in myelin compaction (Boison et al., 1995; Duncan et al., 1987; Jahn et al., 2009).

Paradoxically, PLP/DM20-deficient mice show little myelin abnormality (Klugmann et al., 1997), manifesting compact myelin of appropriate thickness though with abnormally condensed IPLs. Yet despite relatively normally appearing myelin, both PLP-deficient mice and patients with PLP null mutations show evidence of axonal injury (Griffiths et al., 1998), suggesting a role of PLP in axonal support. In addition, DM20 may have a more specific role in brain development, as it is expressed early during ontogeny, before glial cells are formed (Ikenaka et al., 1992). PLP and DM20 are differentially expressed in the central and peripheral nervous systems, with predominance of PLP in the central nervous system (CNS), and DM20 in the peripheral nervous system (PNS) (Pham-Dinh et al., 1991). Yet myelin protein zero (MPZ) is the major protein constituent of myelin, and PLP and DM20 together comprise less than 1% of peripheral myelin protein (Garbern et al., 1999b), likely accounting for the minimal effects of PLP mis-expression on peripheral nerve function and relative rarity of significant PNS dysfunction in PMD

E. Genetic heterogeneity of PMD

PLP gene duplications are the most common cause of Pelizaeus-Merzbacher disease (Mimault et al., 1999; Sistermans et al., 1998), accounting for 50 to 75% of all clinically manifest mutations (Garbern et al., 1999a) and commonly yielding the classic PMD phenotype. Point mutations account for another 20% of cases, and are associated with highly variable phenotypes; these can vary from clinically mild to severe connatal forms (Cailloux et al., 2000). The clinical heterogeneity of PLP1 point mutations is a function of mutation site: point mutations within exons can result in non-synonymous alterations in protein sequence, while mutations in introns can result in disruption of splice site junctions as well as of intronic regulatory sequences (Garbern et al., 1999a). PLP1 deletions are less common, accounting for <5% of identified cases; paradoxically, these are associated with milder phenotypes, thus predisposing these cases to under-diagnosis. Peripheral neuropathy is a common feature of PLP1 deletion-associated syndromes, although less severe CNS disease may have the effect of increasing the diagnostic yield of symptomatic peripheral neuropathies in these patients (Garbern et al., 1997) (Figure 2).

Duplications

The PLP1 gene is in Xq22.2, chromosomal segment prone to genomic instability, and in particular to submicroscopic duplications. This region harbors several low copy repeats proximally and distally, which may contribute to the heterogeneity of its rearrangements. There is variation in the position of both proximal and distal breakpoints (Inoue et al., 1999; Woodward et al., 1998), although distal breakpoints tend to cluster around low-copy repeats (LCRs) (Regis et al., 2008; Woodward et al., 2007). Duplications have been reported to range from 100kb to 4.6Mb in size, and may include not only PLP1, but also neighboring genes (Inoue et al., 1999; Lee et al., 2006). PLP1 duplications appear to result from non-homologous pairs between the proximal and distal breakpoints (Lee et al., 2006), a

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mechanism that differs from non-allelic homologous recombination between low-copy repeats that flank the rearranged genomic sequence (Woodward et al., 2007).

The pathological mechanisms that lead to PMD caused by PLP1 duplications can be assessed in mice expressing supernumerary copies of PLP1, since the latter is a highly conserved gene across species. These mice have been reported to manifest PLP over-expression ranging from 1.3–7.4 fold of normal (Kagawa et al., 1994; Readhead et al., 1994). The mice suffer severe demyelination, myelin swellings and decreased PLP/DM20, as well as deficits in associated myelin proteins including MBP, CNP and Myelin Oligodendrocyte Glycoprotein (MOG). Some studies show evidence of oligodendrocyte maturation arrest and eventual cell death, associated with swelling of the Golgi apparatus (Kagawa et al., 1994). Other reports have noted that despite their decreased myelin, oligodendrocytes proceed to differentiate, but ultimately extend fewer processes than normal oligodendroglia (Gow et al., 1998).

The severity of disease of these mice is proportional to the level of PLP overexpression. Increased levels of PLP may lead to sequestration of cholesterol in the lysosomal compartments, resulting in abnormal cellular trafficking of lipid rafts and sphingolipids that are normally sorted out of the Golgi compartments, and this in turn may result in oligodendrocyte injury and early oligodendroglial death (Garbern, 2006; Simons et al., 2002). There is axonal preservation in these models, but not in another mutant with a larger PLP1 duplication that includes PLP1 as well as neighboring genes commonly involved in affected patients (Clark et al., 2013). The latter model results in a milder phenotype that more closely resembles classic PMD, but is associated with axonal injury as well as vacuolation in the white and grey matter. PLP duplications can also lead to splicing abnormalities with changes in the PLP1/DM20 ratio, as shown in fibroblasts from affected patients (Regis et al., 2009).

Missense mutations

Point mutations in the PLP1 gene can lead to missense, nonsense, frame-shift and silent mutations; missense are the most common. Missense mutations of the PLP1 gene cause protein misfolding, consequent endoplasmic reticulum (ER) retention and disruption of the normal trafficking of PLP to the cell surface (Gow et al., 1994; Gow and Lazzarini, 1996; Woodward, 2008). Accumulation in the ER leads to activation of the unfolded protein response (UPR) and consequent oligodendrocytic death (Gow and RR, 1996; Gow et al., 1998; Southwood et al., 2002). The efficiency of degradation of proteins retained in the ER may contribute to variability in phenotype. Although some proteins are rapidly cleared by the ER associated degradation complex, others are resistant to this process, resulting in induction of the unfolded protein response (UPR) and consequent cell death (Roboti et al., 2009). Another proposed mechanism responsible for the inefficient degradation of PLP is the formation of stable oligomers that can accumulate in the ER, and that fail to be subsequently cleared and degraded. Such oligomers are normally formed only once the protein reaches the cell membrane, likely achieving its native conformation at the site where its function is required (Swanton et al., 2005).

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Mutations affecting different domains of the protein result in distinct pathological presentations. Adding to the difficulty in correlating specific genotypes with disease phenotype, there is significant variability in the functional severity of nearly-adjacent mutations affecting the same PLP structural domains. Normal assembly of the transmembrane (TM) domains is necessary for surface expression of PLP (Cailloux et al., 2000), as truncated proteins with fewer than the four TM domains are retained in the ER. Interestingly, co-expression of two PLP half-mers, which in isolation are retained in the ER, result in reassembly and consequent PLP expression at the cell surface, suggesting an ability of PLP protein fragments to self-align their TM domains (Dhaunchak et al., 2011). The second extracellular domain (EC2) is a particularly common mutant genotype, in part dues to its two disulfide bridges, inner Cys184-Cys228 and outer C201-C220. Substitutions of the cysteine residues in the extracellular loop of PLP result in significant misfolding and trafficking within the ER, likely by altering the globular structure of EC2 arranged by these 2 bridges, an effect more pronounced for the inner than outer loop (Dhaunchak and Nave, 2007). Even when EC2 mutations do not affect the cysteine bridge residues, the unpaired cysteine residues can yield PLP retention in the ER, possibly by crosslinking of sterically exposed cysteine residues with PLP itself or other proteins. More so, some mutations in other amino acids within the EC2 domain that result in ER retention can be rescued by substituting the outer cysteine residues (Dhaunchak and Nave, 2007).

Deletions and null alleles

PLP1 deletions likely originate by non-homologous end joining, possibly in areas with low copy repeats, although the breakpoints can also be created by homologous recombination as well (Inoue et al., 2002b). Deletions of PLP1 are associated with a loss of PLP function, which is most commonly associated with the development of hereditary spastic paraplegia type 2. Interestingly, the complete absence of PLP and DM20 proteins yields a milder phenotype than those associated with either PLP1 duplications or point mutations. Similarly, animal models lacking PLP due to a deletion affecting exon and intron 1, have no overt phenotype and are long-lived (Klugmann et al., 1997). Structurally, myelin compaction remains evident, although an abnormally condensed intraperiod line has been noted (Klugmann et al., 1997), and loose myelin wraps become evident, apparently as a result of Wallerian degeneration (Garbern et al., 2002a). However, despite relatively intact myelin, PLP1 deletion can be associated with marked axonal injury, which is both progressive and length-dependent (Garbern, 2006). This observation suggests a direct role for PLP in the maintenance of axonal viability, and thus in oligodendroglial support of neuronal function. Impairment of transport of growth factors or mitochondria to the distal axon, making it more susceptible to damage, has been hypothesized (Garbern et al., 2002a). There are, however, significant differences between transgenic PLP1 null mice and patients with PLP1 null phenotype, in that myelin abnormalities are more evident in humans than in their murine counterparts. It has been speculated that PLP might be more important for myelin compaction and stabilization in humans, but not for myelin formation; as such, early postnatal myelin formation might be expected to be normal, with deletion-associated myelin failure occurring only later, as the result of poor homeostatic myelin maintenance (Garbern et al., 1997; Klugmann et al., 1997). Other factors that can account for these inter-species differences in the apparent role of PLP might include the caliber of the axonal cohort,

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alternative compensatory mechanisms in rodents, and the relatively short follow-up of the animal models, since the development of myelin dysfunction may be slow and hence delayed in appearance (Garbern et al., 1997).

F. Genotype-phenotype correlations

Duplications

PLP duplications are the most frequent PLP mutations, accounting for 60–70% of all cases (Sistermans et al., 1998). PLP1 duplications are generally associated with classic forms of PMD (Inoue, 2004) but there is some heterogeneity in the phenotype (Hoffman-Zacharska et al., 2013) with connatal forms not infrequently reported (Shimojima et al., 2010; Wolf et al., 2005b).

Duplications are variable in size and do not disrupt PLP1 coding, suggesting that affected neighboring genes may contribute to the phenotype (Woodward et al., 1998). Indeed, the extent of the duplicated genomic segments does not predict the clinical phenotype (Regis et al., 2008; Shimojima et al., 2010); other factors such as the genetic background of the individual, the PLP1 dosage and abnormalities in neighbor genes affected by rearrangement may play a role in the phenotypic outcome. Like mouse models with increased copies of PLP1, in which disease severity is proportional to the increased gene dosage (Kagawa et al., 1994; Readhead et al., 1994), patients with PLP triplications have a more severe phenotype than those with duplications, and manifest a course similar to connatal disease (Shimojima et al., 2012). Patients with PLP1 triplications also have epilepsy (Wolf et al., 2005b), an otherwise unusual feature of PMD. Nonetheless, five copies of the PLP1 gene resulted in a similarly severe phenotype to gene triplications, suggesting that there is a limit in this increased copy number effect (Wolf et al., 2005b). However, since patients with only two copies of PLP1 can also manifest a severe phenotype (Shimojima et al., 2010), other modifiers must also play a role determining the phenotype.

Deletions

Relatively few cases with PLP1 deletions have been described, most of which include small deletions. They may involve two other neighbor genes but typically not more. The phenotype is generally milder and accompanied by peripheral neuropathy, a hallmark not usually present in most cases of duplications and missense mutations. Complete deletion of PLP1 gene was initially described in a family with a milder phenotype (Raskind et al., 1991), later confirmed to have concurrent asymmetric slowing in nerve conduction studies (NCSs) (Garbern et al., 1997). Several partial deletions have been described involving the exon 1 and the surrounding promoter/intronic sequences, the exon 6 to the to 3’ UPR (Combes et al., 2006) and the second extracellular domain of the protein (Grossi et al., 2011). Although some of these share severe phenotypes that resemble classic PMD, they still course with the characteristic neuropathy. Large deletions are uncommon in males, perhaps because they may be incompatible with life. However in heterozygous females large deletions can result in spasticity and significant cognitive and behavior impairments (Matsufuji et al., 2013; Yamamoto et al., 2014). In some cases the phenotype may be related to deletions in neighbor genes such as the interleukin 1 receptor accessory protein-like 2

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(IL1RAPL2) gene and the clustered brain expressed X-linked (BEX) genes (Yamamoto et al., 2014).

Hereditary spastic paraplegia type 2 and null PLP phenotype

Mutations resulting in null expression of PLP, either by deletions (Matsufuji et al., 2013), nonsense mutations or mutations affecting PLP specific splicing, result in milder phenotypes with a spectrum that can range from uncomplicated or complicated hereditary spastic paraplegia type 2 (HSP2) to PLP null phenotypes and the distinction between both can often be ambiguous. HSP2 is an allelic condition to PMD, characterized by a milder phenotype with slowly progressive spasticity of the lower extremities, that can be isolated or, more frequently, complicated by the presence of cerebellar symptoms and cognitive impairment (Garbern, 2006). Pathologically, there is evidence of degeneration of the pyramidal tracts. Symptoms typically start within the first decade, although in some cases the onset can be delayed (Sivakumar et al., 1999). Most of the described cases have point mutations within the PLP specific region in exon 3B, resulting in abnormal PLP expression but spared DM20 (Hodes et al., 1998; Osaka et al., 1995; Saugier-Veber and Munnich, 1994; Sivakumar et al., 1999), but other mutations in exon 2 (Noetzli et al., 2014), exon 4 (Kobayashi et al., 1994) and exon 7 (Suzuki et al., 2011) that do not spare DM20 have also been described. The mechanism that dictates the phenotype is yet unclear but hypothesized to result from proteins that are better cleared by the ER and less prone to activate a strong UPR, resulting in improved oligodendrocyte differentiation and myelin formation and maintenance compared to other mutation types.

Insight into the pathogenesis of HSP2 has been gleaned from studies of the *rumpshaker* mouse, a PLP1 mutant with a mild phenotype. *Rumpshaker* carries a point mutation in exon 4, similar to that of humans with HSP2. Enhanced proteosomal degradation, rather than decreased synthesis, results in decreased levels of PLP/DM20 protein, although a small proportion of the protein can still be correctly assembled and incorporated into the myelin sheath (McLaughlin et al., 2006). However the mechanism of disease cannot be fully explained by decreased PLP levels in the membrane, since the PLP null mouse actually has more myelin than the *rumpshaker* model.

The PLP1 null phenotype can result from point mutations affecting the PLP1 open frame (Garbern et al., 1997), PLP1 start codon (Sistermans et al., 1996) and others that result in absent PLP/PMD. Mutations predicted to result in the absence of the PLP isoform often course with peripheral nervous system involvement (Garbern et al., 1997; Grossi et al., 2011), with axonal or mixed axonal and demyelinating neuropathy (Grossi et al., 2011). PLP expression in Schwann cells may be more crucial than DM20 for normal PNS myelination, as point mutations in the PLP specific domain resulting in truncated PLP but normal expression of DM20 result in peripheral neuropathy (Shy et al., 2003). Peripheral involvement has not been reported in patients with PLP1 duplications (Grossi et al., 2011; Shy et al., 2003), except in a case of duplication downstream to PLP1, possibly by affecting PLP1 regulatory sequences resulting in decreased PLP expression (Lee et al., 2006). Length-dependent axonal injury is seen particularly in PLP null phenotypes caused by deletions or nonsense mutations, and is not associated with significant demyelination. Electron

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microscopy (EM) evidence of axonal spheroids and axonal degeneration has been demonstrated in patients with no PLP expression, as well as in rodent models with PLP1 null mutations (Garbern et al., 2002b). Low NAA levels by MRS correlated with pathology, and can be used as a marker for axonal injury (Garbern et al., 2002a). Neuronal degeneration is also present in some cases, with thalamic and cerebellar involvement. Loss of Purkinje cells was indeed observed across patients with different mutation types, possibly explaining the high incidence of ataxia seen in this disorder (Sima et al., 2009).

Missense mutations

Missense mutations result in highly heterogeneous phenotypes (Roboti et al., 2009), encompassing the most severe (connatal) and mildest forms (HSP 2) (Fig 3). Our review of all cases with point mutations reported to date disclosed a majority of severe phenotypes (42%), followed by classic forms (38%) and mild phenotypes in lower percentage (20%). Severe forms of PMD are generally associated with substitution of highly conserved amino acids but no other strong associations have been found between phenotype and the type or location of the mutation within the PLP1 gene and PLP/DM protein (Fukumura et al., 2011). Mutations affecting the PLP specific sequence or splicing and not DM20 have been associated with milder phenotypes (Cailloux et al., 2000; Grossi et al., 2011; Hodes et al., 1998; Saugier-Veber and Munnich, 1994) and mutations that disrupt both PLP and DM20 trafficking have been long identified to result in more severe phenotypes (Gow and Lazzarini, 1996). However, severe phenotypes were also described in males and also females with a nonsense mutation in the PLP-specific exon 3B (Marble et al., 2007), in patients with synonymous point mutations involving the last nucleotide of exon 3B (Hobson et al., 2006), and in patients with a mutation in exon 3B resulting in abnormal splicing affecting PLP but not DM20 (Lassuthová et al., 2013). The deleterious effects of the predicted aberrant PLP protein may dictate variable degrees of cytotoxicity and consequent phenotype. Seizures are again uncommon with few non refractory cases reported in patients with missense mutations (Cailloux et al., 2000).

Mutations in non-coding areas and splice variants

Mutations in non-coding regions that disrupt the normal splicing of PLP1/DM20 precursor RNA are another cause of PMD (Hobson et al., 2006). Mutations in intronic splice donor, branch and acceptor sites can all result in functionally null or truncated proteins. Mutations in intronic enhancers, that bind transcription factors that regulate transcription, or interact with the splicing machinery to regulate alternative splicing, can also lead to disease (Hobson et al., 2006). Several non-coding PLP mutations have been described in patients with PMD, most commonly in intron 3; depending upon site these mutant phenotypes are clinically heterogeneous, leading to presentations ranging from severe connatal to milder and later-onset forms of PMD (Figure 3). Of note, no mutations affecting the DM20 splicing site have yet been described; these may be embryonic lethal, since DM20 is produced early in development and may have a broader function than PLP (Campagnoni and Skoff, 2001). In contrast, mutations affecting PLP splice sites are more common, and those lead to a reduction or absence of functional PLP protein (Hobson et al., 2006). Most of the intronic mutations described to date are in intron 3, affecting splice donor sites (Hobson et al., 2000; hubner et al., 2005; Lassuthová et al., 2013; Shy et al., 2003), acceptor sites (Cailloux et al.,

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2000; Cailloux et al., 1996; Shimojima et al., 2010), and splice regulatory sequences (Hobson et al., 2006; Taube et al., 2014). Those regulatory sequences can be located far from the splice sites, and their interaction may be necessary for normal PLP1/DM20 splicing (Taube et al., 2014). Intragenic mutations affecting other PLP introns have also been reported; a common result of such splice site mutations is skipping of the preceding exon (Hobson et al., 2000), as seen in intron 5 (Osaka et al., 1999) and intron 6 mutations (Hobson et al., 2000). These intronic mutations are associated with a wide range of clinical phenotypes, that thus far defy systematic correlation to clinical severity (Fig 3).

Female carriers

As an X-linked disorder, PMD is clinically apparent in boys, but is carried by their mothers and may be so by their sisters. Obligate female carriers are generally asymptomatic. However, some females do show signs of disease; indeed, the first description of affected females appeared in Merzbacher's initial report (Merzbacher, 1910). The risk to carrier females of developing clinical disease is greatest for nonsense indel or null mutations, followed by some missense mutations, and is less so for duplications (Hurst et al., 2006). Nonetheless, all mutation types have been reported in symptomatic females, including duplications (Inoue et al., 2001), deletions (Inoue et al., 2002b; Raskind et al., 1991), nonsense (Hodes et al., 1998) and missense mutations (Hodes et al., 1995). Interestingly, affected females are noted more frequently in families characterized by mild disease in males (Hurst et al., 2006), although that is not always the case (Marble et al., 2007). This may reflect a compensatory effect, as in the developing brains of heterozygous females, oligodendrocytes expressing mutant PLP may be more likely to die and be replaced by oligodendrocytes expressing wild-type PLP1 gene, assuming stochastic random X inactivation of the two alleles in any given cell. According to this scheme, in mild disease (caused by PLP1 nonsense point mutations and deletions), the PLP mutant oligodendrocytes may not die or be competitively dominated by wild-type cells; these brains might instead accumulate PLP-mutant oligodendroglia over time, resulting in clinical phenotypes. In contrast, in the brains of carriers of severe mutations (PLP1 missense mutations and duplications), favorable skewing of the population towards healthy cells occurs precisely, if paradoxically, because of the selective death of mutant PLP oligodendrocytes (Marble et al., 2007; Woodward et al., 2000).

When symptomatic, females tend to manifest a milder, later-onset phenotype than their related males, more commonly characterized by late onset progressive spasticity and cognitive decline, although their presentations can vary considerably among different families. Less commonly, early onset symptoms followed by slow developmental improvement has been reported in cases with duplications and missense mutations. A more severe phenotype, with earlier onset and significant cognitive impairment has also been reported (Hodes et al., 1995; Warshawsky et al., 2005), as has been an atypical presentation that can resemble primary progressive multiple sclerosis (Yamamoto et al., 2014). The basis for these more severe phenotypes is unknown, but it has been speculated that unfavorably skewed X inactivation could account for some of those cases with early onset that show improvement overtime. In such cases, most oligodendrocyte precursors would fail to properly differentiate and myelinate, resulting in clinical disease, followed by the selective

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emergence of wild type oligodendrocytes as the mutant cells die, leading to consequent clinical improvement (Marble et al., 2007).

Although PMD is a characteristic X-linked disorder inherited exclusively by the mother, the origin of *de novo* mutations differs among mutation types. Unlike sporadic point mutations that occur with the same frequency in males and females gametes, sporadic duplications occur preferentially on the paternal chromosome during spermatogenesis. The mechanism responsible for such male bias in duplications may involve unequal sister chromatid exchange, as proposed in other X linked disorders (Mimault et al., 1999).

G. Approach to the genetic diagnosis of PMD

Initial screening should target PLP1 duplications, as those are the most common genetic cause of PMD. Sensitive and specific quantitative methods to detect duplications include quantitative polymerase chain reaction (qPCR) (Inoue et al., 1999), multiplex ligation-dependent probe amplification (MPLA) (Warshawsky et al., 2006) and chromosomal microarray that includes this gene segment (Garbern et al., 2002b; Hobson and Kamholz, 2013). These methods also detect deletions within the PLP1 gene. Although duplications may also be screened by interphase fluorescent *in situ* hybridization (FISH), this method has limitations and should not be used in isolation. FISH may not separately detect duplicated regions close to each other, can give false positive results when sequences adjacent to PLP1 but not including the PLP1 gene are duplicated (since as the FISH probes include sequences flanking PLP1) and has limited ability to detect more than two copies per cell (Garbern et al., 2002b; Warshawsky et al., 2006). An advantage of FISH, however, is that it can detect mosaicism for duplications and deletions, as well as PLP1 duplications in the rare event of its integration into other genomic locations (Garbern et al., 2002b; Warshawsky et al., 2006), so FISH analysis is commonly performed after detecting increased PLP1 copy numbers to determine if the duplication is a direct or insertional event (Hobson and Kamholz, 2013) (Figure 4).

If no PLP1 duplications or deletions are found, sequence analysis of the 7 exons and splice donor and acceptor sites should next be performed; these may be expected to diagnose 10–20% of cases. Full sequencing of intron 3 is also included in most labs, since mutations in splice regulatory sequences have been so commonly identified as pathogenic (Figure 4). Although small intragenic deletions/insertions can be detected by sequencing, larger all exon deletions/duplications are not detected, thus sequencing should not be performed prior to duplication/deletion analysis, unless in the case of families where a point mutation is known. If a point mutation is detected, the predicted effect of the mutation can also be investigated from previously described genotype-phenotype correlations, or by predictive algorithms, such as Ensembl Variant Effect Predictor tool (http://useast.ensembl.org/info/docs/tools/vep/index.html), Mutpred (http://mutpred.mutdb.org/about.html), Skippy (http://research.nhgri.nih.gov/skippy/index.shtml) and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/index.shtml). As noted, genotype-phenotype associations between PLP1 mutation and clinical PMD have proven difficult, with considerable variability even within the same family, rendering prognosis difficult. Nonetheless, some general rules may be formulated: point mutations affecting highly conserved amino acids tend to result in severe disease; more

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than two copies of the PLP1 gene are associated with more severe phenotypes than PLP1 duplications (Wolf et al., 2005b); point mutations within exon 3B tend to result in milder phenotypes; and mutations resulting in null expression of PLP/DM20 also tend to result in milder disease, often with peripheral manifestations (Cailloux et al., 2000).

Differential diagnosis

If the testing scheme described above proves unrevealing, then alternative diagnoses should be considered. Mutations in the gap junction gamma-2 gene (GJC2), which expresses connexin 47 (Cx47), has been associated with an autosomal recessive form of PM-like disease (PMLD), designated as hypomyelinating leukodystrophy 2 (HLD 2, MIM# 608804), and more commonly called PMLD-1. This condition resembles PMD, and can be clinically indistinguishable from it. In addition, a milder phenotype designated spastic paraplegia autosomal recessive type 44 (SPG44, MIM# 613206), which resembles HSP2 and also involves Cx47 mutation (Biancheri et al., 2013; Garbern and Hobson, 2002; Henneke et al., 2008; Jellouli et al., 2013), may also be considered. The GJC2 gene locates to 1q42–1q43, and encodes connexin 47, which is highly expressed in oligodendrocytes. In a large multiethnic cohort of patients with PMD-like disease, Cx47 mutations described only 8% of cases, although this fraction was higher in consanguineous families (30%) and symptomatic female (16%) (Garbern et al., 2002b; Henneke et al., 2008; Hobson and Garbern, 2012).

Besides the Cx47 deficiencies, other hereditary leukodystrophies should also be considered in the evaluation of possible PMD cases. A number of other hypomyelinating leukodystrophies have been described (reviewed in (Boespflug-Tanguy, 2013; Hobson and Garbern, 2012; Powers, 2004; Steenweg et al., 2010; Vanderver et al., 2015), the clinical descriptions of which are beyond the scope of this review, but whose similarities to PMD, especially to later-onset cases of classical PMD, suggest caution in the use of the term PMD-like. Important points in assessment and testing that can help in differentiating among these conditions include eye examination, to assess the possible concurrence of congenital cataracts (Boespflug-Tanguy, 2013; Zara et al., 2006); dental examination, to assess the possibility of hypomyelination with ataxia and hypodontia (Wolf et al., 2005a; Wolf et al., 2014); and thyroid studies, abnormalities of which suggest the delayed myelination noted in the MCT8 thyroid transporter deficiency of the Allen-Herdon-Dudley syndrome (Schwartz et al., 2005).

Prenatal counseling and diagnosis should always be offered to affected families. Amniotic or chorionic villus cells can be used to test point mutations, duplications and deletions of the PLP1 gene when there is a positive family history of PMD. FISH probes have been used to successfully diagnose PLP1 duplications prenatally from amniotic fluid cells (Inoue et al., 2002a; Woodward et al., 1999), and from chorionic villus cells using quantitative fluorescence multiplex PCR (Regis et al., 2001). Preimplantation genetic diagnosis is also possible, and has successfully selected unaffected embryos with the consequent birth of healthy children (Verlinsky et al., 2006).

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H. Treatment options and efforts

No treatment currently exists for PMD. The use of pharmacological agents to lower PLP1 expression, promote PLP incorporation into the cell membrane, and enhance the ability of the endoplasmic reticulum to clear misfolded proteins, have all been suggested as potential therapeutic avenues, based on pathway extrapolations and animal studies. Administration of lonaprisan, an antagonist of the progesterone receptor, resulted in decreased mRNA overexpression, increased number of myelinated axons, and functional motor improvement in PLP overexpressing mice (Prukop et al., 2014). A cholesterol-rich diet administered to mice with increased PLP copy numbers resulted in increased oligodendrocyte survival, increased axonal caliber, a decrease in the large lysosomes typically present in affected cells, and functional improvement of mice already manifesting disease. In the presence of increased cholesterol, excessive PLP appears to be directed into myelin assembly, resulting in decreased intracellular PLP and cholesterol alike (Rudolphi et al., 2012). Similarly, curcumin (diferuloymethane), a polyphenol dietary compound derived from the curry spice turmeric, has been tested in *msd* mice, who carry a missense mutation A242V in the PLP1 gene, given its postulated role in improving the clearance of misfolded proteins via inhibition of the endoplasmic reticulum Ca^{2+} ATPase (Yu et al., 2012). In this mouse model of PMD, oral curcumin resulted in prolongation of survival by 25%, and decreased the rate of oligodendrocytic apoptosis, although with no apparent myelin reconstitution or motor recovery (Yu et al., 2012). None of these agents has yet been evaluated in clinical trials.

Studies such as these highlight the need to develop better models for screening potential therapeutic agents, whether in vitro or in preclinical animal models. Over the past several years, induced pluripotential cells (iPSCs) have evolved as disease-derived tools by which to assess both biological mechanisms and cell-specific therapeutic responses on a patient-specific basis. iPSCs and their derived glial progenitors and oligodendrocytes may be produced from affected patients, and used to study the effect of different mutations *in vitro* and *in vivo*. In fact, iPS cells generated from PMD patients with missense mutations have been successfully differentiated into oligodendrocytes with recapitulation of PMD pathology (Numasawa-Kuroiwa et al., 2014); these oligodendrocytes have manifested ER stress and death, validating the hypothesis that protein misfolding-associated ER stress might trigger oligodendroglial loss in PMD (Gow and Lazzarini, 1996; Gow et al., 1998).

Besides these efforts to develop disease-modifying therapies based on targeting proteolipid protein metabolism and/or degradation, strategies are under development for using cell therapeutics to directly restore lost or deficient myelin in PMD patients. Intracerebral transplantation of glial cells may be a particularly promising approach towards this end, in that this approach attempts to rescue the disease phenotype outright, by replacing mutant cells with oligodendrocytes carrying a normal copy of the PLP1 gene. The ability of human glial and oligodendrocyte progenitor cells to rescue a murine model of congenital hypomyelination (Goldman et al., 2012; Wang et al., 2013; Windrem et al., 2008), and similarly, of neural stem cells to produce glial progenitors and their daughter oligodendrocytes in vivo (Uchida et al., 2012), provided the basis for the first phase 1 clinical trial in PMD. This was accomplished by the intracerebral delivery of serially-propagated human neural stem cells into four patients with connatal PMD (NCT01005004)

(Gupta et al., 2012). The transplanted boys received immunosuppressive therapy for 9 months, and all carried missense mutations in transmembrane (TM) domains, two in TM2 and two in TM4. The trial investigators reported a favorable safety profile at 1-year after transplantation, by both clinical and radiological evaluation, but the myelination competence of these neural stem cell grafts remains to be clearly established. Nonetheless, the investigators plan long-term evaluation of both safety and efficacy, using developmental, clinical, radiographic, and neurophysiological outcome measures, all of which will be assessed as long as 4 years after transplant (NCT01391637). Other cell therapeutic strategies have also been advocated for the treatment of PMD. Umbilical cord blood transplantation (UCBT) was performed in two boys with PMD, one caused by PLP1 duplication in the other by a deletion in intron 5, resulting in aberrant splicing. Neither the mechanism nor potential benefits of such a treatment strategy are clear, but at least no adverse events were reported at 1 or 7 years after transplant in these patients. That said, the patients' lack of clinical deterioration during this period and mild increase in MRI-assessed myelination were well within the range of the natural development of PMD children (Wishnew et al., 2014), the clinical variability among whom is significant.

Besides using tissue-derived sources of neural stem cells and glial, progenitors, embryonic stem cells and iPSCs may be similarly used as sources of allogeneic glial progenitor cell grafts (Wang et al., 2013). Given the advent of gene editing techniques, correction of mutant patient derived iPSCs followed by their glial induction and intracerebral transplantation might permit autologous transplantation to be a feasible option in the future (Fox et al., 2014). Indeed these studies, involving evaluation of novel cell therapeutics in small numbers of patients with a genetically heterogeneous and phenotypically diverse rare disorder, highlight the need for appropriate controls in evaluating treatment efficacy. As exciting as these new strategies of cell therapy might be in the development of new treatments for afflicted children, and as strong as the preclinical animal data may be suggesting their likely therapeutic benefit, we will be unable to establish which treatments indeed provide significant benefit, whether that benefit might outweigh the risks inherent in cell therapy, and whether the durability of their benefits justifies the effort, until we can claim a better understanding of the genotype-dependent natural history of PMD.

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This chapter is dedicated to the memory of our friend and colleague Dr. James Y. Garbern, who contributed so profoundly to our understanding of Pelizaeus-Merzbacher disease, and whose dedicated care of patients and families suffering its effects provided inspiration for us all.

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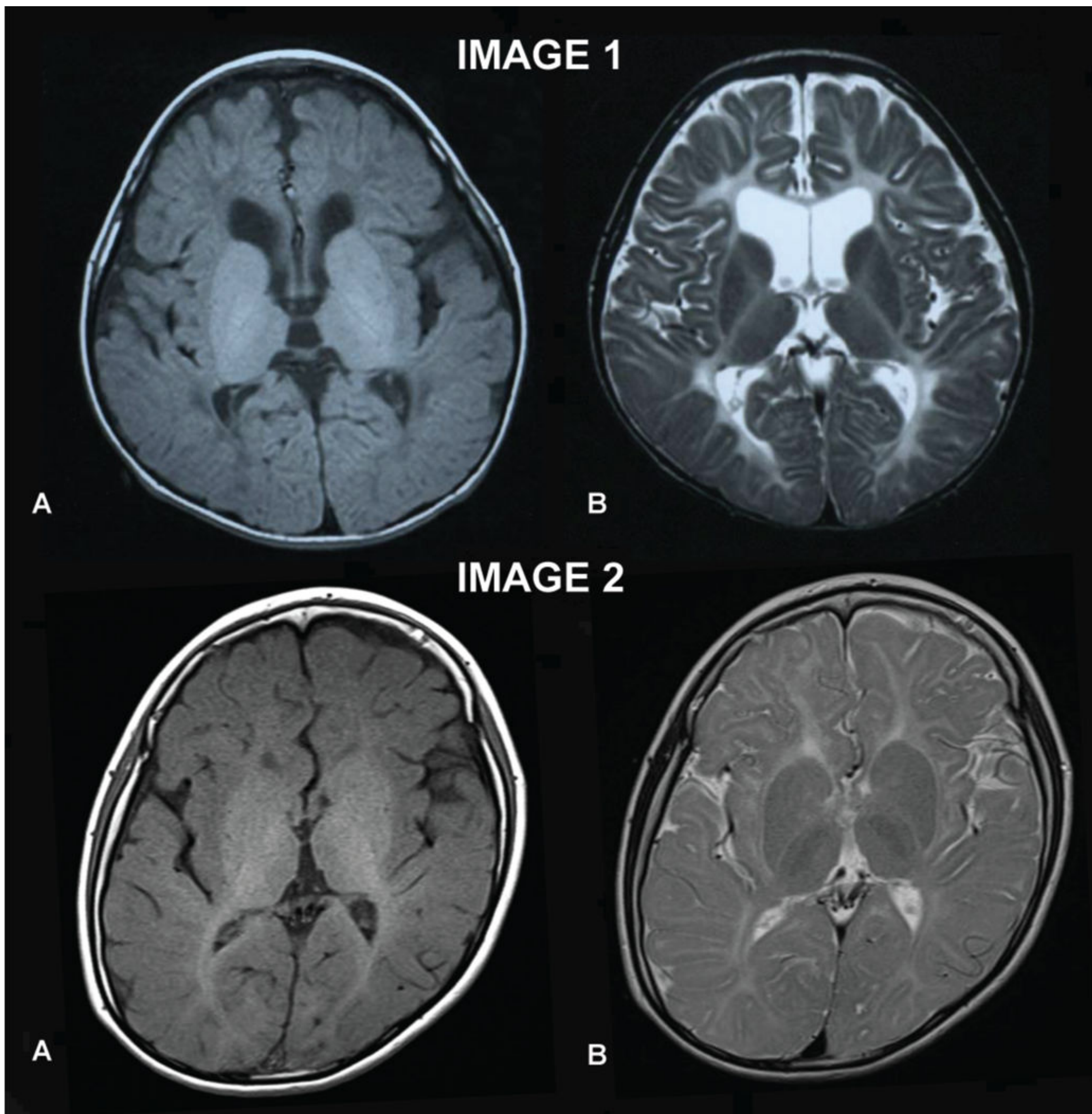

Figure 1. Brain MRI findings in patients with connatal and classic PMD

Image 1 Brain MRI of a 9 year-old boy with connatal PMD caused by a missense mutation, V209D. There is total absence of myelin, both in T1 (A) and T2 (B) weighted-images; the patient did not achieve any motor milestones.. **Image 2** Brain MRI of a patient with classic PMD at age 10. In contrast to image 1, some myelination is evident, although diminished for age, seen as high signal intensity in the T1-weighted image in the posterior limb of the internal capsule, optic radiations and lateral thalamus (A), though with little corresponding

signal attenuation on T2-weighted sequences, all indicative of hypomyelination. From Takanashi *et al.* (Takanashi et al., 1999), with permission.

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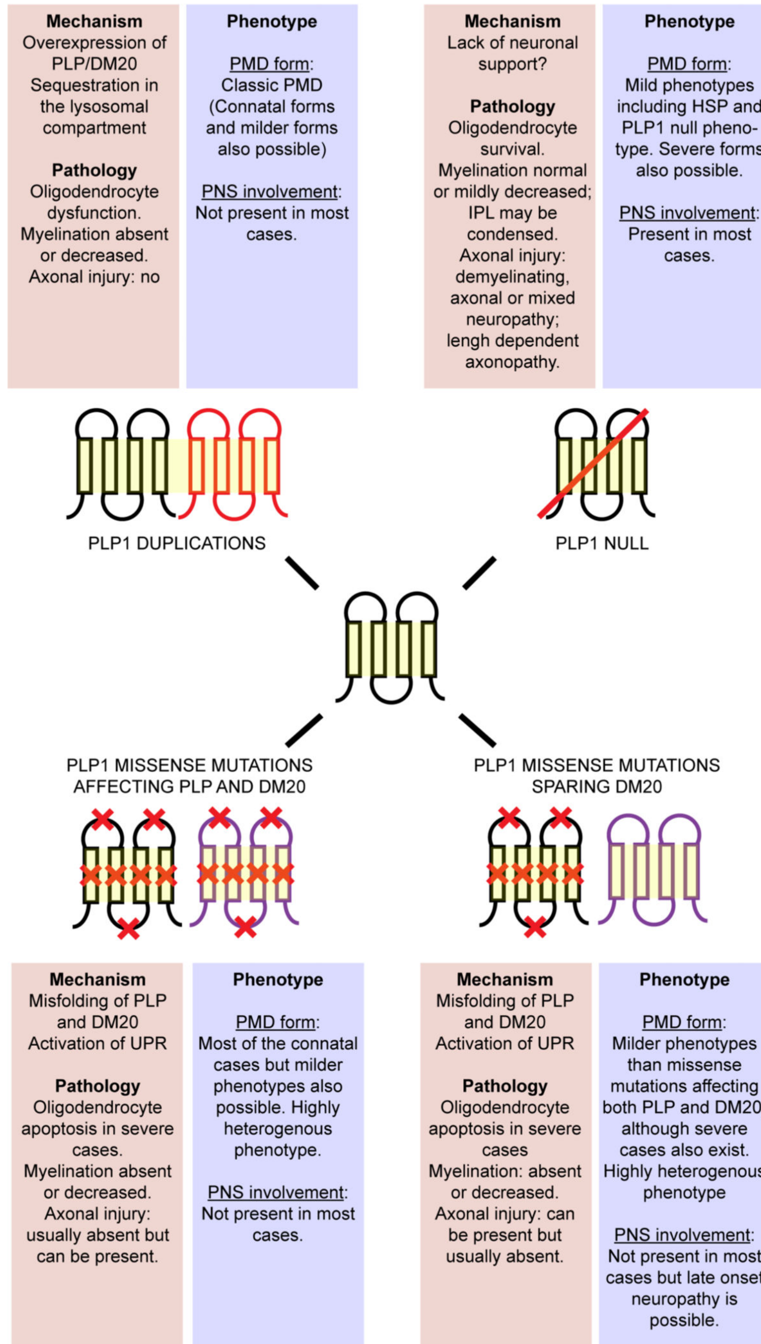

Figure 2. Major types of PLP1 mutations

PLP1 mutant genotypes include PLP1 duplications, PLP1 null mutations (due to deletions or nonsense point mutations), and missense mutations. For each type, the proposed cellular mechanism and pathology findings are described (orange boxes). The clinical PMD phenotype and the presence or absence of peripheral nervous system (PNS) involvement is also detailed for each mutation type (purple boxes).

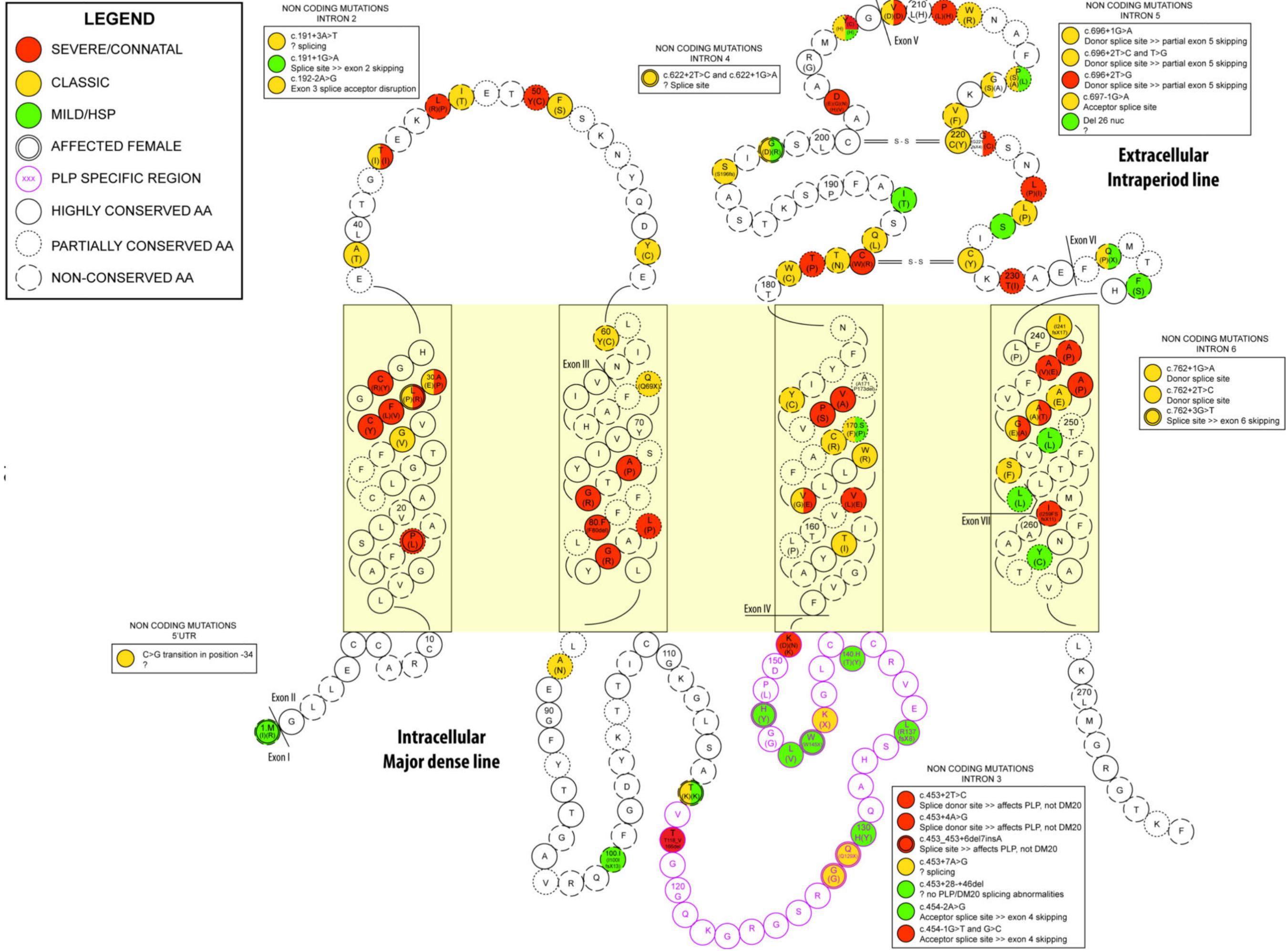

Figure 3. PLP point mutations predict aspects of clinical phenotype

The color code represents degrees of severity with red for severe cases, yellow for classic cases and green for mild cases. Severe cases were defined as patients who failed to achieve any motor or defined as stage 0 per the Cailloux *et al* classification (Cailloux et al., 2000); when undetermined, cases of neonatal onset or with rapidly progressive phenotype resulting in no motor achievements were included. Classic PMD was used for patients who achieved some motor milestones but not independent gait or defined as stage 1, 2 or 3 per Cailloux *et al* classification. Mild PMD included cases of late onset symptoms with achievement of independent gait or defined as stage 4 per Cailloux *et al* classification; it included most subjects with HSP phenotype. In some cases the distinction among these 3 levels was not obvious and the best attempt to match in the category that would best fit the description was attempted. Undetermined cases were coded without a color. The contour of each protein codes for the conservation of the amino acid across different species (human, chicken, mouse, trout and squalus sharks) as defined by Cailloux *et al*, with full line coding for conserved amino acids (90–100%), short dashed line for partially conserved amino acids (60–89%) and large dashed line for non-conserved amino acids (<60%). Amino acids in purple represent the PLP specific amino acids, removed in the DM20 spliced form. Affected

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females were shown with a double contour. Intronic mutations and severity code are illustrated in separate tables. Nomenclature: Amino acid residue numbering was derived from the human PLP protein (genebank-EMBL Accession Number NP_000524.3). Methionine encoded by the translation initiation site is numbered as residue 1; mutations described using the old nomenclature were corrected accordingly.

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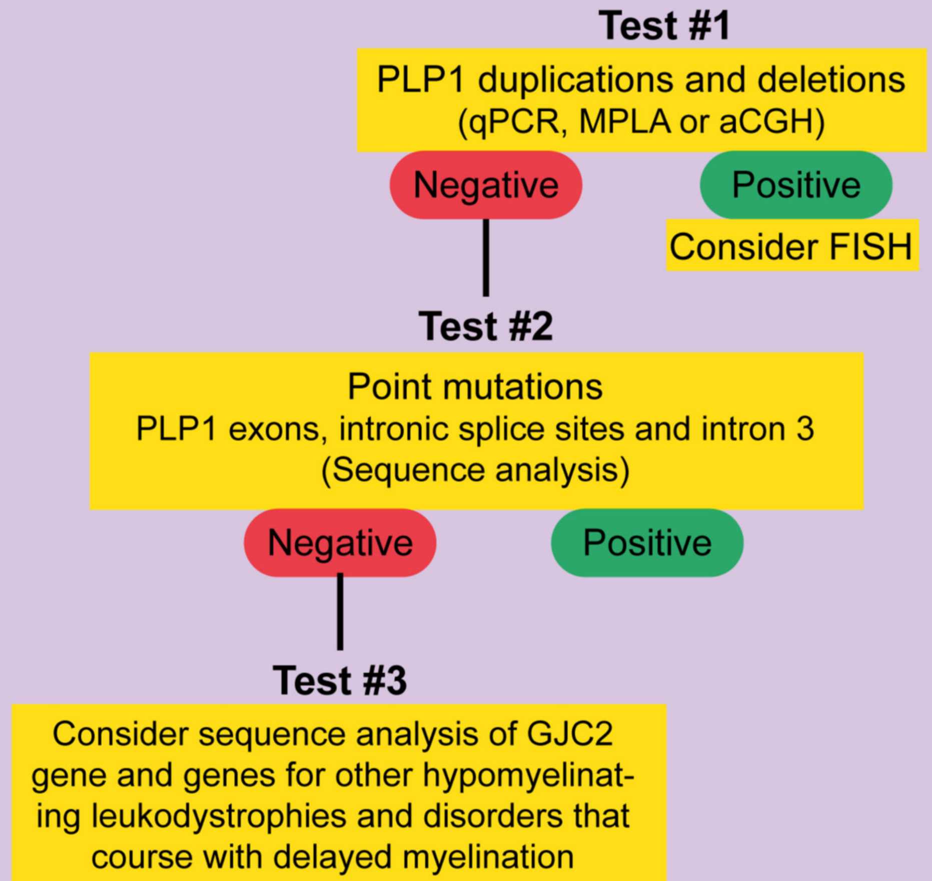

Figure 4. Genetic testing for PMD/HSP2

Molecular testing for PMD/HSP2

Testing for duplications should be the first step in the diagnosis of PMD, as it is the most common mutation. Quantitative multiplex polymerase chain reaction (qPCR), multiple ligation probe analysis (MPLA), or array comparative genomic hybridization (aCGH) may all be used to detect copy number changes. aCGH has the advantage of determining the extent of the region that is duplicated. If positive, subsequent metaphase fluorescent in situ hybridization (FISH) analysis can determine if the increased copy number localizes to PLP1 or if it is inserted in another part of the genome. Metaphase FISH has low sensitivity for initial testing, as it fails to detect small duplications. As a result, if duplication/deletion analysis is negative, sequence analysis of the PLP1 gene should follow. All exons and the intronic flaking regions containing the donor and acceptor splice sites should be evaluated. Most laboratories will add sequencing of intron 3, in which most intronic mutations have

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been described. If negative, alternative diagnoses should be considered, as outlined in Section G.